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cGMP signalling in plants; from enigma to main stream

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Abstract

All living organisms communicate with their environment and part of this dialogue is mediated by secondary messengers such as cyclic Guanosine Mono Phosphate (cGMP). In plants, most of the specific components that allow production and break-down of cGMP have now been identified apart from cGMP dependent phosphodiesterases, enzymes responsible for cGMP catabolism. Irrespectively, the role of cGMP in plant signal transductions is now firmly established with involvement of this nucleotide in development, stress response, ion homeostasis and hormone function. Within these areas, a number of consistent themes is slowly emerging where cGMP may be particularly relevant and these include regulation of cation fluxes, for example via cyclic nucleotide gated channels, and in stomatal functioning.

Many details of signalling pathways that incorporate cGMP remain to be unveiled. These include down stream targets other than a small number of ion channels, in particular cGMP dependent kinases. Improved genomics tools may help in this respect, especially since many proteins involved in cGMP signalling appear to have multiple and often overlapping functional domains which hampers identification on the basis of simple homology searches. Another open question regards the topographical distribution of cGMP signals; are they cell limited? Does long distance cGMP signalling occur and if so, by what mechanisms? The advent of non-disruptive fluorescent reporters with high spatial and temporal resolution will provide a tool to accelerate progress in all these areas. Automation can facilitate large scale screens of mutants or the action of effectors that impact on cGMP signalling.

Keywords

cGMP, CNGC, guard cell, ion homeostasis, signalling, stress

Introduction

Cells and organisms must have means to perceive, transduce and respond to multiple stimuli, which may originate internally or derive from the outside environment. Stimuli include physical cues such as changes in temperature, light or precipitation, but also biological agents such as elicitors, hormones or action potentials. On the other end of the chain, responses typically involve changes in gene expression and post-translational modifications in effector proteins such as glycosylation, ubiquitination and phosphorylation. Stimulus specificity is relatively easy to recognise in the panoply of different primary signals and in the ultimate responses. However, most, if not all, of these signal pathways will rely on the action of (cytoplasmic) intermediates in the form of secondary messengers that function as 'telephone exchanges' by passing on multiple incoming signals to specific downstream targets.

The number of secondary messengers is relatively small with some of the most ubiquitous ones consisting of simple ions such as Ca^{2+} (Dodd et al. 2010) lipids like Inositol-3-phosphate (IP3) (Munnik and Testerink 2009) and the cyclic nucleotides cyclic Adenosine Monophosphate (cAMP) and cyclic Guanosine Monophosphate (cGMP) ((Newton and Smith 2004; Martinez et al. 2007). However, this list is continuously extending and other small molecules like nitric oxide (NO), reactive oxygen species (ROS) and Cyclic ADP-ribose (cADPR) or Nicotinic acid adenine dinucleotide phosphate (NAADP) form further secondary messengers (Bailey-Serres and Mittler 2006). The small number of secondary messengers relative to the multitude of stimuli implies there must be coding information to maintain specificity, a phenomenon that is only partially understood for Ca^{2+} but remains an enigma for most other intermediates. In the case of Ca^{2+}, both frequency and amplitude coding affects Ca^{2+} binding proteins that show different affinities. For example, Ca^{2+} dependent kinases with different binding constants will be activated in response to different levels of cytoplasmic Ca^{2+}. For the cyclic nucleotides 3',5' cAMP and 3',5' cGMP different signal amplitudes have been observed but how these translate into specific activation of downstream targets is as yet unclear.

Well established in animals, protozoa and prokaryotes, the signalling role of the cyclic nucleotide mono phosphates (cNMPs), cAMP and cGMP in plants was long doubted but the drastically improved analytical techniques have unequivocally shown the presence of both cAMP and cGMP in several plant species (Newton and Smith 2004) . Furthermore, there is now sound evidence for biological activity of both nucleotides, but especially cGMP, in a range of cellular and physiological processes. For example, cGMP has been implemented in abiotic and biotic stress responses, developmental programmes, ion homeostasis and many other essential processes.

Cyclic Nucleotide Biochemistry and Biosynthesis

One of the main contributors to the scepticism surrounding plant cNMPs was the lack of evidence for specific proteins to catalyse the biosynthesis and degradation of these nucleotides. To a large extent, this is no longer true. For example, in the last decade, several proteins having adenylate or guanylyl cyclase activity have been identified based on a non-canonical sequences and the first guanylyl cyclase (GC) in plants (AtGC1) was discovered and shown to have a GC activity *in vitro* (Ludidi and Gehring 2003). Today, both cytosolic and membrane localised GCs have been described in plants (Figure 1). The latter include the receptor nucleotide cyclase Phytosulfokine receptor 1 (PSKR1), the pathogen peptide1 receptor (atPepR1), the brassinosteroids receptor atBRI1 and the wall associated kinase like 10 (AtWAKL10) whereas two soluble GCs are AtGC1 and the NO induced AtNOGC1. It has to be noted though that for some of these receptors, the *in vitro* GC activity is relatively low (typically ~1000-fold less than that observed for mammalian soluble GCs) whereas for others GC activity per se has been questioned (Ashton 2011; Bojar *et al.* 2014). On the other hand, some of the membrane bound Leucine rich repeat receptors like WALK10 or PSKR1 have been shown to have activity that is similar to that of membrane bound GC receptors in animals with a Vmax of around 2000 $nmol.min^{-1}.mg^{-}1$ (Meier *et al.* 2010; Kwezi *et al.* 2011). Interestingly, although in mammalian proteins the GC domain is usually found adjacent to the kinase domain, in plant proteins the GC domain is encapsulated within the kinase domain, suggesting one domain exhibits both kinase and GC activity. Alternatively, cGMP could act as a competitive inhibitor for the kinase site and hence is important for the regulation of the kinase activity of these membrane receptors. BRI1 for example, was found to be phosphorylated in response to cGMP (Marondedze *et al.* 2016) .

cNMP signals must be terminated which is partly achieved by reduction in cNMP levels. In animals, cGMP is catabolised by cyclic nucleotide phosphodiesterase (CN-PDE), an enzyme that has also been reported to be present in prokaryotes and eukaryotes. Mammals count 20 CN-PDEs. Surprisingly, although CN-PDE activity has been reported in several plant preparations, the protein(s) that carries out this function in plants is still unknown. Its identification is made difficult by the lack of canonical sequences found in other organisms in plants. Interestingly, CN-PDE inhibitors such as IBMX or sildenafil, used in other eukaryotes, show activity in plant systems, suggesting that similarity in their reaction centres is present (Ishioka and Tanimoto 1990) .

Uncertainty about the role of cNMPs in plants was also fuelled by inadequate analytical techniques. It appears the cNMP levels in plants are significantly lower than those in animal cells although some of this can be explained by the large central vacuole that occupies most plant cells. Because of the low concentrations of cNMPs in plants, detection methods with high sensitivity were needed. Improved analytical techniques based on HPLC-MS fit the bill in this respect and unequivocally proofed the presence of both cGMP and cAMP in plant cells. In addition, techniques based on immunoassays can be applied to plant tissue. However, both of these methods are expensive and lack spatial and temporal resolution. The development of fluorescent protein and luciferin based reporters for cNMPs (Isner and Maathuis 2011; Wheeler *et al.* 2013) means that cNMP levels can now be detected non-invasively. A luciferase dependent reporter where a cGMP responsive promoter OPTX is fused to the gene encoding luciferase is now available (Wheeler *et al.* 2013) and able to detect changes in cGMP concentration of less than 100 nM. In plants, reporters that correlate fluorescence to cGMP levels are available based on cGMP binding domains from mammals and the circulated permutated GFP (Isner and Maathuis 2011). It has a dynamic range of 20 to 20000nM, a Kd of 180nM. Using a range of different techniques, GMP has been detected in a number of plant species, including barley, tobacco and Arabidopsis, and in different tissues, Although the data vary considerably, amounts are typically 1–50 pmol per gram Fresh Weight (gFW) which roughly translates into cytoplasmic concentrations of cGMP in the range of 10–500 nM (Maathuis 2006). Furthermore, during perception of stimuli, these cellular cGMP concentrations have been shown to increase 5- to 10-fold (e.g. Isner et al. 2011; Donaldson et al., 2004; Durner et al., 1998) resulting in concentrations up to 5000 nM, values that map perfectly on the properties of GFP based reporters. Thus, endogenous reporters are now available with high resolution in detection level, space and time. Furthermore, reporters can be fused with different targeting signals to localise probes to specific organelles thus achieving subcellular resolution which was previously impossible.

cGMP Targets

With an increasing biochemical basis in place that is capable of driving the biosynthesis and breakdown of cGMP in plant cells, there still remains the enigma of how dynamic changes in cytoplasmic cGMP concentration are translated into sensible responses. A classic set of downstream effectors of cNMP signalling in both animals and fungi are cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively). In spite of a dearth of plant genomic data, as yet, there is precious little evidence of bona fide PKGs in plant cells at the sequence level. Similarly, at the biochemical level evidence is scarce and limited to pharmacological approaches e.g. (Szmidt-Jaworska *et al.* 2009; Nan *et al.* 2014) based on the use of Rp-8-Br-cGMP, a non-metabolised membrane permeable cGMP analogue. However, such studies have to be interpreted with great caution: Rp-8-Br-cGMP is not specific and is likely to irreversibly block any protein that normally binds cGMP. So what else could function as cGMP translator? Plant genomes do contain sequences that encode proteins with both a cyclic nucleotide binding domain and a protein kinase domain (Martinez-Atienza *et al.* 2007). Thus, PKA- and PKG-type kinases may function in plants but simply have low homology to their animal counterparts. Other than kinases, proteins with a clearly defined cyclic nucleotide binding domain consist mainly of ion transporters, with cyclic nucleotide gated channels (CNGCs) the most prevalent amongst these (Figure 2). CNGCs function cation transport and Ca^{2+} signalling (Demidchik and Maathuis 2007). They are gated via binding of cAMP or cGMP to a cyclic nucleotide binding domain (CNBD) near the C-terminus. Additional control over gating is provided by a calmodulin binding domain which in plants is located at the C-terminus and partly overlaps the CNBD. CaM binding lowers CNGC activity by preventing cyclic nucleotide binding. CNGCs have a generalised predicted structure of six transmembrane domains, S1-S6, with a pore domain (P loop) between S5 and S6. Most plants appear to have large CNGC gene families and CNGC isoforms have been shown to be important in various biotic and abiotic stresses (Kaplan *et al.* 2007). CNGCs are mostly non-selective and Ca^{2+} permeable (Demidchik and Maathuis 2007), making them ideal vehicles to

translate cGMP signals into Ca^{2+} signals. In addition to CNGCs, many Shaker-type potassium channels, and a few sodium-proton antiport mechanisms contain cyclic nucleotide domains that may be involved in protein regulation.

The Role of cGMP in Basic Plant Functions

Information about the exact nature of cGMP binding proteins would greatly facilitate the characterisation of physiological cGMP roles. Although this is often unclear, cGMP has been shown to partake in many physiological processes. One main area where cGMP has been implicated is stomatal functioning but unfortunately with often contradictory roles. cGMP was previously hypothesised to be involved in stomatal opening as a component of the response to natriuretic peptides (Pharmawati *et al.* 1998; Pharmawati *et al.* 2001) or to auxins (Cousson 2003). Mechanistically, cGMP might open stomata by modifying guard cell turgor (Turek *et al.* 2014) via the plant natriuretic receptor AtPNP, which carries guanylyl cyclase activity. In contrast, in pea guard cells, cGMP has been involved in stomatal closure induced by ABA or NO (Desikan *et al.* 2004). Later studies suggested that cGMP acts upstream of Ca^{2+} in H_2O_2 induced stomatal closure in an ABA independent pathway (Dubovskaya *et al.* 2011). The upstream position of cGMP relative to Ca^{2+}, could be mediated by plasma membrane localised CNGC5 and CNGC6 channels, which were found to be involved in cGMP-induced Ca^{2+} inward rectifying current (Wang *et al.* 2013). As mentioned above, cGMP has also been shown to be involved in NO-induced stomatal closure (Neill *et al.* 2002) possibly with NO acting as agonist to GC.

Although many of these findings appear contradictory, cGMP could induce both stomatal opening and stomatal closure, depending on the exact conditions. Such a dual role of cGMP has been recently proposed after discovery of the cGMP derivative 5-nitrosyl-cGMP. The latter is induced by ABA and NO in a ROS dependent manner (and hence promotes stomatal closure), whereas the non-nitrosylated form cGMP has been shown to play a role in stomatal opening in the dark (Joudoi *et al.* 2013). Joudoi et al. (2013) also showed that the NO dependent GC NOGC1 failed to close stomata in response to NO. Other cGMP derivatives are capable of inducing stomatal closure, such as 8-mercapto-cgmp which is involved in H2S-induced stomatal closure (Honda *et al.* 2015). cGMP has also been shown to play a role in cell protoplasts expansion, which could be linked with stomatal aperture regulation. A scenario where the GC activity of polypeptide phytosulfokine (PSKR1) leads to activation of CNGC17 to allow cation influx, while a functional coupling between these proteins and plasma membrane located proton pumps such as AHA1 and AHA2, culminates in increased H^+ efflux and hence cell expansion (Ladwig *et al.* 2015). Cell expansion can be a prerequisite of stomatal opening (Wang *et al.* 2007).

Plants cells have to regulate their ion concentrations, which is critical for development and physiological roles. Ion homeostasis is another prime example of cGMP involvement. Ion homeostasis largely depends on mechanisms that control the permeability, abundance or activity of intrinsic membrane proteins such as channels and transporters. There is evidence that cGMP acts at all these different levels: It regulates expression of many genes, including that of many (mainly monovalent) root membrane transporters (Maathuis 2006). Furthermore, it directly affects the activity of voltage-independent ion channels (Maathuis and Sanders 2001) and that of CNGCs (see above). cGMP may also regulate the activity of root channels and carriers by changing protein expression and via posttranslational regulation: Expression of proteins involved in stress response and ion transport was specifically affected in microsomes exposed to cyclic nucleotides (Ordonez et al. 2014) while the phosphorylation status of transporters changed within minutes after cGMP exposure (Isner *et al.* 2012). At the whole tissue level, cGMP is known to reduce Na^+ influx in several species and as such relieves salt stress (Maathuis and Sanders 2001). Experimentation on intact roots also shows that H_2O_2-induced K^+ and Ca^{2+} fluxes are affected by cGMP (Ordonez et al. 2014). According to work by Li et al. (2014), cGMP is also an upstream factor of the ethylene pathway involved in the response to salinity by maintaining a higher Na^+/K^+ ratio and by increasing plasma membrane H^+-ATPase activity. Natriuretic peptides were found to have a role in water and salt balance via atPNP-R1 and atPNP-A, which were found to interact with each other and have a guanylyl cyclase activity (Turek and Gehring 2016).

Around 20 years ago Penson et al. (1996) showed that cGMP plays a crucial role in the GA dependent pathway in the barley aleurone layer. GA increases cGMP levels, which in turn activates alpha-amylase (Penson *et al.*

1996). Further indications for a role of cGMP in germination stem from observations of an NO-induced stimulant of lettuce seed germination (Beligni and Lamattina 2000), presumably via the stimulatory activity of NO on GCs. Similarly, in wheat, cGMP mimics the effects of NO on the alpha-amylase activity (Wu *et al.* 2013) whereas in barley cGMP together with GA promote germination but also reduce the ABA sensitivity during seed germination (Gomez-Cadenas *et al.* 2001).

Not surprisingly the second messenger cGMP has been implicated in the relay of several hormones, some of which were briefly mentioned above. There is probably a strong link between brassinosteroids and cGMP. Brassinosteroids regulate plant development and many aspects of plant physiology. Brassinosteroid pathways start with its binding to the membrane receptor kinase BRI1. BRI1 receptor recruits BRI1-ASSOCIATED RECEPTOR KINASE1 (BRAK1) and gets dissociated from its inhibitor BRI1 KINASE INHIBITOR1 (BKI1) before getting phosphorylated. Subsequent phosphorylation of downstream factors allow further transduction of the signal. Interestingly, and based on a degenerated motif found in animal GCs, a GC domain has been found within the kinase domain of BRI1. When using an endogenous cGMP sensor no change in cGMP level was observed in mesophyll protoplasts (Isner *et al.* 2012) but BR was shown to increase cGMP levels at the root tip (Zhao *et al.* 2013). The exact nature of the link between BR and cGMP awaits clarification but could unfold via CNGC2 (allelic to DND1) to catalyse an elevation of cytosolic Ca^{2+}. Ca^{2+} in turn might upregulate the expression of INDOLE-3-ACETIC ACIDINDUCIBLE1 and PHYTOCHROME B ACTIVATION-TAGGED SUPPRESSOR1 (Zhao *et al.* 2013).

As previously mentioned, cGMP has been linked to auxins in stomatal aperture regulation. cGMP has also been linked to auxins in the context of root development, mainly because of its link with NO. Explants replete in auxin showed 3.5 times more NO than those depleted for the hormone (Pagnussat *et al.* 2003). Also, root development induced by IAA and NO was reduced by the GC inhibitor LY83583 but reversed by application of the membrane permeable cGMP analogue 8-Br-cGMP. However, the adventitious root development process also appears to have a cGMP-independent pathway involving MAPKs, suggesting that the interaction between auxin and cGMP is more complex (Pagnussat *et al.* 2004). Adventitious rooting is linked to H_2O_2 and H_2O_2 promotes rooting via a Ca^{2+}, MAPK, and cGMP dependent pathway (Li and Xue 2010). The GC inhibitor LY83583 was shown to prevent elevation of cytosolic Ca^{2+} in this context and the CDPK-mediated signal induced by auxins and NO (Lanteri *et al.* 2006). More precisely, cGMP has been proposed to act on the auxin gradient through the modulation of PINs (auxin transporters) localisation and expression. Thus, cGMP may regulate lateral root formation by modulating polar auxin transport. cGMP also mediates NO and auxin transport induced gravitropic curvature in soybean roots (Hu *et al.* 2005). Finally, cGMP levels increase after IAA treatment in protoplasts (Isner *et al.* 2012).

As mentioned before, studies on barley germination inferred a strong link between gibberellin (GA) and cGMP (Penson *et al.* 1996) with GA generating an increase in cGMP levels which were instrumental in activation of α-amylase gene transcription and subsequent enzyme secretion. Similarly, Teng et al. (2010) showed that both cGMP and GA are required for seed germination in *Arabidopsis*, suggesting that cGMP may be a common component in plant seed germination. Direct measurement using endogenous reporters showed that cGMP levels rose within seconds after exposure of rice protoplasts to GA (Isner *et al.* 2012) while a reporter gene fused to a cGMP-induced promoter showed an enhanced signal after adding five GA response elements to the promoter (Wheeler *et al.* 2013) .

Isner et al (2012) showed that ABA induced cGMP in rice protoplasts, seconds after treatment. Salt and osmotic stress are known to increase ABA levels and induce a rapid increase in cGMP levels in *Arabidopsis* seedling (Donaldson et al., 2004). cGMP content of pea epidermis and *Arabidopsis* guard cell fragments transiently increases following ABA treatment and (Neill *et al.* 2008) while the same investigators (Neill *et al.* 2002) showed that cGMP was required for ABA induced closing of stomata. Other studies (Joudoi *et al.* 2013) detected a six-fold increase in 8-nitro-cGMP levels after treatment with 10µM ABA. Challenge with GC inhibitors prevented ABA-induced stomatal closure. All these observations point to an intricate relationship between cGMP and ABA however, the mechanistic details and sequence of components are clearly lacking. The use of mutants and genetics may improve this situation (for example, cGMP was placed upstream of ABA

in the stomatal closing response by Dubovskaya et al (2011) since cGMP-induced stomatal closure was abolished in the *abi1* mutant) but will require robust phenotypes which is not always forthcoming.

Compared to ABA, relatively little evidence points to a connection between the gaseous hormone ethylene and cGMP. Ethylene plays a role in plant developmental as well as a biotic and abiotic stresses. By using membrane permeable cGMP, Li et al (2014) showed that cGMP induces an increase of ethylene of around 50%, whereas the inhibition of GCs reduced ethylene levels by about 30%. Although purely correlative, these findings indicate that cGMP is involved in ethylene production. The cGMP improved ion homeostasis in *Arabidopsis* and its associated protective effect in salt stress disappeared when either plants were exposed to the ethylene biosynthesis inhibitor aminooxyacetic acid, or when an ethylene receptor mutant (*etr1*) was used. This shows that cGMP is likely a downstream component of the ethylene signalling pathway. However, the molecular interactions are unknown.

Plant peptide signalling (Figure 3) is a relatively new era of research. Peptides regulate growth, cell development, root elongation, fertilisation and are involved in plant-pathogen interaction (Wheeler and Irving 2010). Peptides are typically released as pro-peptides into the apoplast and, once cleaved, bind to LRR kinases. Interestingly, 3 pathways that are initiated by peptides (CLAVATA3 (CLV3), phytosulfokine (PSK) and peptide PEP3) use cGMP as a secondary messenger to link with downstream signals (Qi *et al.* 2010; Kwezi *et al.* 2011; Chou *et al.* 2016). Several of these receptors contain a GC domain within their kinase domain (i.e PEPR1, CLV1). Although the receptor of plant natriuretic peptide hasn't been identified yet, PNP, within seconds, induces a cGMP level increase. Similarly, CLV3 induced a time dependent cytosolic cGMP response in the root tips. The Phytosulfokine (PSK) Receptor was shown to be capable of cGMP synthesis upon binding of its peptide effector Phytosulfokine, and so did AtPepR1 when bound by its agonist the Atpep3 peptide.

Clavata1 is a receptor expressed in the shoot apical meristem that maintains stem cells at their undifferentiated stage by inhibiting WUSCHEL and FANTASTIC FOUR. The inhibition of WUCHSEL by CLV3 peptide was shown to be inhibited by Ca^{2+} channel blockers as well as guanylyl cyclase inhibitors. This inhibition was also observed in the dnd1 and dnd2 mutants which lack the cyclic nucleotide gated channel 2 and 4 respectively. This suggests that when CLV3 peptide binds to the CLV1 receptor in the meristem, cGMP levels increase and activate CNGC2 channels, leading to an increase of cytoplasmic Ca^{2+}. The elevated Ca^{2+} levels cause altered expression of WUS and FAF2 and eventually change the fate of the meristem cell.

Phytosulfokine (PSK) is a ubiquitous sulphated pentapeptide that binds the LRR receptor PSKR1 or PSKR2. PSK promotes cell growth, acts in the quiescent centre cells of the root apical meristem, contributes to funicular pollen tube guidance and differentially alters immune responses depending on the pathogen (Sauter 2015). As mentioned above for CLV1 and PEPR1, PSKR1 has GC activity in addition to its kinase function. *In vivo*, the overexpression of PSKR1 in leaf protoplasts raised the endogenous basal cGMP levels over 20 times and GC activity was induced after binding of the sulphated effector PSK-alpha to the receptor, and not when a non-phosphorylated backbone was added (Kwezi *et al.* 2011). In all, these data suggest that plant peptide signalling transduction depends on peptide binding to membrane localised LRRs with dual function. The kinase activity may directly alter cytoplasmic targets but the in many cases the GC activity is required to produce cGMP which catalyses downstream signalling.

Roles of cGMP in plant stress

Like any other living organism, plants are constantly exposed to adverse physical conditions such as extremes in water provision, temperature and salinity. Furthermore, potential pathogens such as viruses, bacteria and fungi are around in all environments while hordes of insects and herbivores that forage on plant material are abundant in most places. Not surprisingly, plants have evolved, and still are evolving, sophisticated defence mechanisms to prevent disease and limit wounding. Such defences rely on constitutive and induced responses, some of them local, while others are systemic and spread around entire organs or even the whole plant.

Evidence of cGMP involvement in biotic stress is mostly indirect. For example, transcription of defence genes such as PAL and PR‑1 has been shown to occur in response to NO with cGMP as an intermediate (Durner *et*

al. 1998). In a different context, elicitor peptides, endogenous compounds which play important roles in evoking plant immunity (see above), are perceived by membrane located receptors which are proposed to have innate guanylyl cyclase activity (Ma *et al.* 2013). Raised levels of cGMP subsequently activate Ca^{2+} permeable channels such as CNGC2, translating cGMP into a Ca^{2+} signal which is not only instrumental in defence gene activation but also provides (via calmodulin) a negative feedback to temper CNGC2 activity.

A more direct link between pathogenicity and cGMP signalling was shown recently for *Arabidopsis* leaves inoculated with *Pseudomonas syringae* (Meier *et al.* 2009). Elevated cGMP levels appeared within 30 minutes of inoculation with cGMP signal-strength varying between virulent and avirulent *Pseudomonas* strains. Whether the observed cGMP signals were directly downstream of recognition of PAMPs (pathogen activated molecular patterns, the primary pathogen-derived stimuli that are perceived by LRR membrane receptors) remains to be unravelled.

Wounding of plant tissue by herbivores evokes complex signalling responses which typically involve the wounding hormone jasmonic acid. Gaupels et al (2016) showed that wounding led to an increase in cellular cGMP, albeit only after 3 hours. It was surmised that this upregulation of cGMP in the phloem tissue of pumpkin is relevant for wound defence reactions but no mechanistic explanation is available as yet. Interestingly, other cGMP isoforms may also be involved in the wounding response as was recently shown by Van Damme et al (2014) who developed an analytical method to accurately measure the 2',3' cGMP isoform and recorded a significant (up to 5-fold) rise in this nucleotide after wounding of *Arabidopsis* leaves.

Like the menace of biotic pests, abiotic hazards are never far away in almost all natural settings. In contrast to responses that are invoked against pathogens which typically involve relatively small numbers of genes, stresses like drought and salinity invariably cause altered activity of hundreds if not thousands of genes. A potential role of cGMP in transcriptional regulation was shown by treatment of roots with membrane permeable cGMP (Maathuis 2006). Though such treatment led to regulation of many genes, there was a clear bias towards transcripts that play a role in abiotic stress responses. A similar bias was found at the protein level in phosphoproteomics studies (Isner *et al.* 2012): data showed that cGMP caused a rapid (within minutes) change in the phosphorylation status of peptides and the affected proteins were more prevalent in the functional categories of abiotic stress, particularly proteins associated with the action of the phytohormone ABA. Indeed, in case of both drought and salinity, a more detailed link has been established between cGMP and stress response (Figure 2): a direct negative effect of cGMP on Na^+ uptake of roots has been shown for several species (Maathuis and Sanders 2001; Essah *et al.* 2003; Rubio *et al.* 2003), whereas direct measurement of cellular cGMP provided evidence for a rapid increase in cellular cGMP after the onset of salt and osmotic stress (Donaldson et al., 2004). Early studies also implicated cGMP in the functioning of plant natriuretic peptides (PNPs), peptidic hormones that systemically affect plant salt and water balance (Wang *et al.* 2011). A link between some PNPs and the drought hormone ABA is well established and relies on production of cGMP, possibly via the membrane located PNP receptor itself which was recently shown to have guanylyl cyclase activity (see above, (Turek and Gehring 2016)). Another mechanism through which cGMP impacts on responses to drought and salinity is via stomatal activity as described in previous sections. However, the extensive evidence for cGMP being involved with both stomatal closure (Dubovskaya *et al.* 2011; Honda *et al.* 2015) *and* stomatal opening (Pharmawati *et al.* 1998; Cousson 2010; Joudoi *et al.* 2013) suggests that small changes in signalling networks can alter the overall bearing of a pathway. The role of cGMP in stomatal regulation is further complicated by conflicting evidence with some labs not finding any impact of cGMP on stomatal aperture (e.g. (Joudoi *et al.* 2013)) but instead suggest that it is the nitrated form of cGMP (NO-cGMP) that is biologically active.

The above studies clearly point to a role of cGMP in plant response to biotic and abiotic stress. Interaction of cGMP with other signalling moieties like NO, peroxide or Ca^{2+}, and with several hormones (e.g. ABA, ethylene, brassinosteroids, auxin) are now well established and have well-defined physiological relevance. However, interpretation and follow-up studies are hampered by a lack of credible downstream targets which for now are largely limited to CNGCs.

Perspectives

It is becoming increasingly clear that plants, like their animal counterparts, have sophisticated signalling systems that comprise multiple interacting players and pathways. In this crowded playing field, cGMP is relatively new in plant signalling and as yet has been shown to play a role in an increasing number of biological phenomena. Progress has been slow due to the difficulty of making accurate routine measurements of cGMP because of secondary metabolites that interfere with analytical assays and the generally low concentrations found in plant tissue (Newton and Smith 2004). With the development of non-disruptive genetic sensors (Isner and Maathuis 2011; Wheeler *et al.* 2013) many of these drawbacks have been removed and it is likely that the number of cGMP functions will increase.

Evidence of cGMP involvement will be more solid if genetic tools improve such as loss and gain of function mutants in catabolic and anabolic steps that allow the experimenter to manipulate cellular cGMP. For this we will need an accurate inventory and functional analysis of the cyclases, phosphodiesterases and other enzymes that contribute to cellular cGMP. Convenient analytical tools such as endogenous reporters will enhance these efforts but also advance automation of cGMP quantification and subsequent high throughput screens of mutants and effect of pharmaceuticals.

Further issues that need urgent attention are the nature of crosstalk between cGMP and other signalling moieties such as Ca^{2+}, lipids and ROS, and spatial dynamics of cGMP signals. In analogy to animals, there is now good evidence in plants that NO signals increase cGMP via guanylyl cyclase activation but is there a direct interaction between cGMP and Ca^{2+}, or cGMP and lipid-based signalling? Cyclic nucleotide gated channels have been suggested to function as nodes that translate cGMP into Ca^{2+} signals (Talke *et al.* 2003) but direct evidence for this is not available. In animals, sphingolipids and phosphatidylinositols lead to the activation of endothelial nitric oxide synthase thereby providing a direct link to NO release and cGMP production. Do similar chains of events act in plant cells?

Some secondary messenger signals can propagate along tissues and organs. This has long been known for Ca^{2+} in response to elicitors or, more recently in response to abiotic stresses like salinity. 'ROS waves' are another example of messenger spreading to multiple cells. Indeed, travel of either (Ca^{2+} or ROS) signal from the initial stimulus to distant cells may depend on the other, though it is likely that electrical signal propagation forms another critical ingredient. Are cGMP signals passed on from cell to cell? As yet, we don't know but one can imagine some feedback loop of cGMP to activation of nitric oxide synthases. There is at least one report that claims nitric oxide synthase in smooth muscle vasculature is activated by cGMP (Inoue *et al.* 1995) . These and other important issues will require access to convenient, cheap and non-disruptive sensors and monitors for cellular cGMP. The use of multiple (fluorescence based) non-disruptive probes, dyes and sensors will greatly help answer these questions and suitable high quantum yield GFP-based sensors are now available for Ca^{2+} (Gilroy *et al.* 2014), cGMP (Isner and Maathuis 2011) and ROS (Gilroy *et al.* 2014). Genetically encoded sensors to monitor NO have recently been reported (Strack 2016). Combinations of these will allow better studies to reveal how dynamics vary between second messengers and which one is placed where in the chain of cause and effect.

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Figure Legends

Figure 1: Overview of anabolic and catabolic pathways for cGMP biosynthesis. The diagram is based on what is known for animal cGMP signalling and biosynthesis. Components that are not clearly defined or as yet absent in plants are depicted in grey. Abbreviations: 'mGC' and 'sGC': membrane bound or soluble guanylyl cyclase; 'NO': nitric oxide; 'PDE': phosphodiesterase. 'CNGC': cyclic nucleotide gated channel; 'PKG' cGMP-dependent protein kinase with 'C' catalytic and 'R' regulatory subunits; 'R' and 'Gs': receptor and Gs subunit of membrane located G-protein; 'NOS': NO synthase; 'CaM': calmodulin; 'PPs': phosphatases.

Figure 2: cGMP and abiotic stress interactions. Evidence for a role of cGMP in abiotic stress derives from observations that, after perception by an unknown receptor (?), cGMP levels are elevated in response to drought and salinity (1). Increased cellular cGMP may also be generated by binding of plant natriuretic peptides (PNPs) that bind to membrane located receptors (2) which are coupled to or have innate guanylyl cyclase activity. Increased cellular cGMP reduces Na^+ uptake (3), and generates a Ca^{2+} signal (4) that may evoke further signalling, for example via calcium sensors like calmodulin (CaM) or calcineurin-B-like proteins (CBL). Loss of cellular K^+ (a frequent side effect of many abiotic stresses) is mitigated (5) by cGMP via inhibition of K^+ leak through outward rectifying channels (ORC) or stimulation of uptake through inward rectifying channels (IRC). cGMP alters transcription of many genes (6) but particularly those involved in stress responses. Activity of further proteins (7), especially K^+ channels and CNGCs, may be involved in ion homeostasis. Abbreviations are as in Fig 1.

Figure 3: Example of pathways involving a LRR receptor kinase with encapsulated guanylyl cyclase. Several transmembrane LRR kinases such as WALK10, PSKR1, CLAVATA1 and BRI1 have been found to contain an encapsulated guanylyl cyclase (GC) domain within their kinase domain. After binding its ligand (paracrine or autocrine), the receptor undergoes dimerization (e.g. PSKR1) or binding with other proteins (e.g. CLAVATA2). GC and kinase domains are then activated. cGMP produced at the receptor site or synthetized from other enzymes may regulate the kinase activity directly. Downstream proteins may include CNGCs, which may generate Ca^{2+} signals and activate calmodulin (CaM) and calcium dependent kinases (CDPKs) that could regulate downstream components such as transcription factor. CaM was shown to interact with some LRR receptors and cGMP can indirectly modulate LRR kinase activity. The LRR kinase can activate transcription factor like WUS or the PM H^+-ATPase (AHA) via phosphorylation. PM; Plasma Membrane.

Figure 1

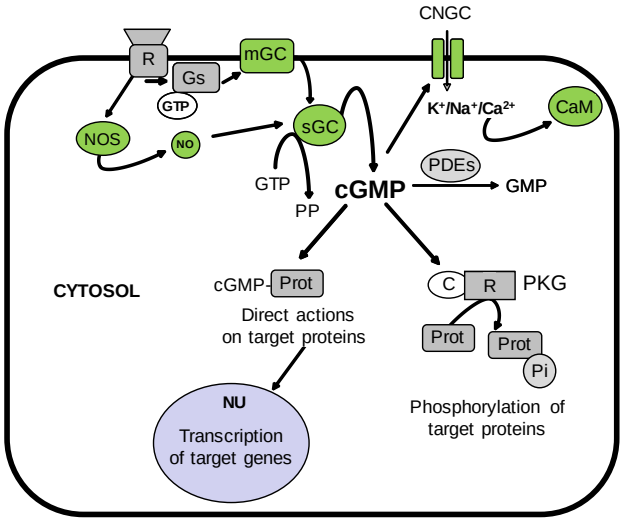

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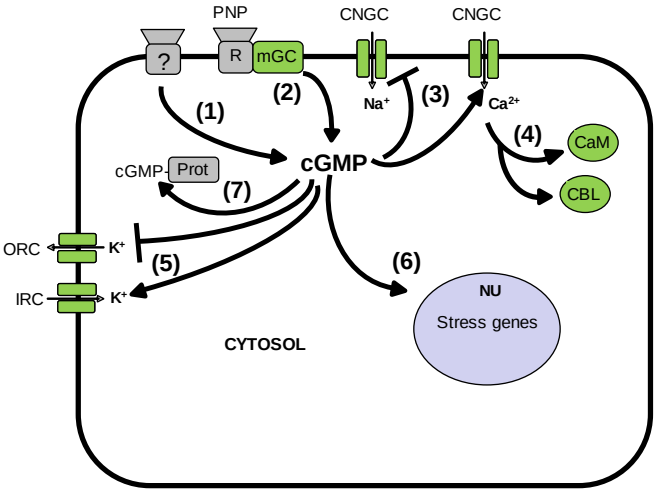
Figure 2



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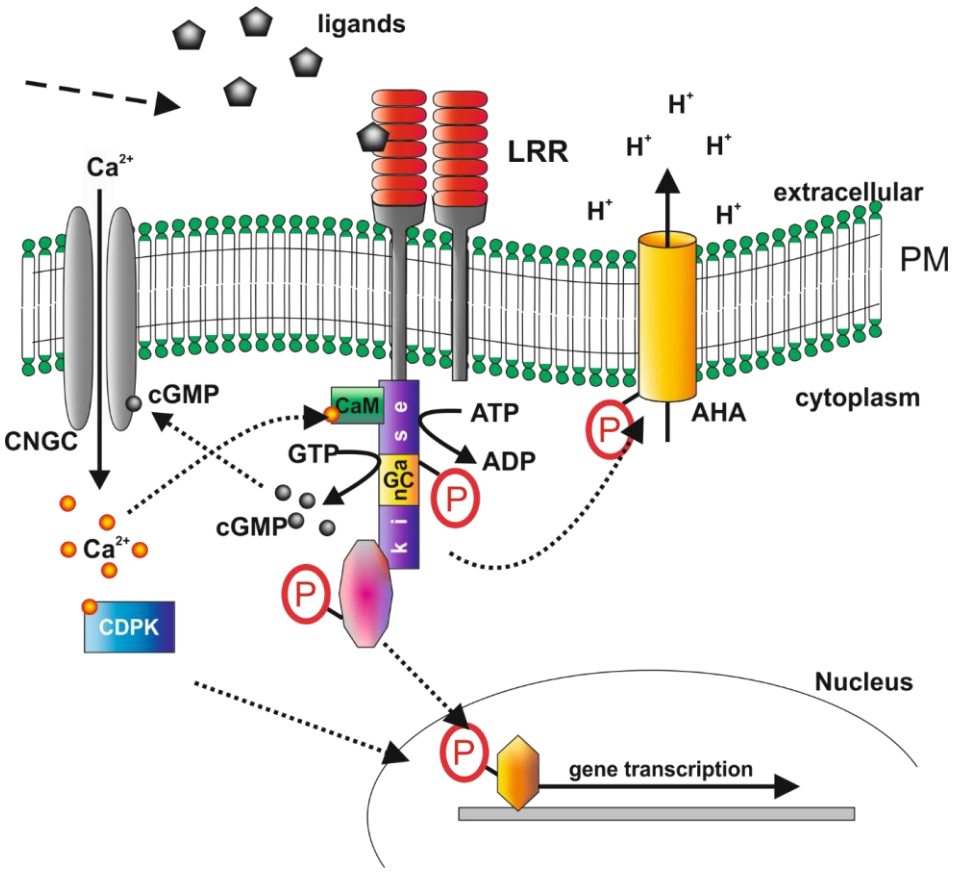

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